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Molecular diagnostic based on 18S rDNA and supplemental taxonomic data of the cnidarian coelozoic *Ceratomyxa* (Cnidaria, Myxosporea) and comments on the intraspecific morphological variation

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Abstract

Ceratomyxa amazonensis is a cnidarian myxosporean originally described with strongly arcuate crescent-shaped myxospores, absence of vegetative stages and infecting *Symphysodon discus*, an important Amazonian ornamental fish in the aquarium industry. As part of a long-term investigation concerning myxosporeans that infect discus fish *Symphysodon* spp. from different rivers of the Amazon Basin, thirty specimens of *S. discus* collected from Unini River were examined. Plasmodial vegetative stages therefrom were found freely floating in the bile of gall bladders from eighteen fish. Mature myxospores were slightly crescent-shaped, measuring 4.72 ± 0.1 (4.52–4.81) µm in length, 24.2 ± 0.4 (23.9–25.3) µm in thickness with polar capsules 2.31 ± 0.1 (2.29–2.33) µm in length and 2.15 ± 0.1 (2.13–2.17) µm in width. Strong morphological differences were observed between the newly isolated myxospores obtained and the previously described *C. amazonensis*; however, molecular assessment, based on 18S rDNA, revealed a high similarity (99.91%), with only a single nucleotide base change. This study provides new data, expanding the original description of the species with a discussion on differences in myxospore-morphology in the context of intraspecific morphological plasticity.

Key Words

Brazil, ceratomyxid, morphological plasticity, ornamental fish, parasitic cnidarian

Introduction

The global aquarium market moves millions of ornamental fish worldwide and is the primary mode for international transport of cnidarian myxosporean parasites (Hallett et al. 2015). As such, there is a fundamental need for constant monitoring to enable diagnosis and timely control of infections by this parasite group in aquarium fish (Mathews et al. 2018). Although the Amazon Basin is amongst the most important sources of wild-caught ornamental fishes in the international aquarium industry (Moreau and Coomes 2007), there are few surveys concerning cnidarian myxosporean infections in Amazonian ornamental fish (Mathews et al. 2015, 2017, 2020a, b). The three recognised species of the discus genus *Symphysodon* Heckel, 1840, in the family Cichlidae, are popular, expensive and widely exploited ornamental fish (Bleher et al. 2007). These neotropical freshwater

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cichlids are endemic to the Amazon Basin and restricted to areas where seasonal flooding occurs (Bleher et al. 2007). The red discus *Symphysodon discus* Heckel, 1840 inhabits lentic aquatic environments, such as floodplains and flooded forests in the lower Rio Negro, upper Uatumã, Unini, Nhamundá, Trombetas and Abacaxis Rivers in Brazil (Amado et al. 2011).

Myxosporeans are endoparasitic microscopic cnidarians with worldwide distributions (Atkinson et al. 2018). With over 2,400 species recorded from aquatic and terrestrial hosts, there is evidence of extensive diversification in and dispersion of this group of parasitic cnidarians (Atkinson et al. 2018). Annelids are the definitive hosts, releasing infective actinospores into the aquatic environment (Fiala et al. 2015). Although virtually all vertebrate groups can be infected, fish comprise the largest number of known secondary hosts (Fiala et al. 2015). Amongst the myxosporeans, species of the genus *Ceratomyxa* Thélohan, 1892 are mostly highly host-specific coelozoic parasites with approximately 300 species that mainly parasitise the gall bladders of a wide range of fish species (Eiras et al. 2018), with some species reportedly generating pathologies in their hosts (Alama-Bermejo et al. 2011, Barreiro et al. 2017). Despite the enormous diversity of fish species in the Amazon Basin, only seven *Ceratomyxa* species have been reported (Eiras et al. 2018, Da Silva et al. 2020). *Ceratomyxa amazonensis* Mathews, Naldoni, Maia & Adriano, 2016 was described as parasitising *S. discus* from the Rio Negro River, Amazonas State, Brazil, with the first published nucleotide sequence of a *Ceratomyxa* species from a strictly freshwater environment (Mathews et al. 2016).

As part of a long-term investigation concerning myxosporeans that infect discus fish *Symphysodon* spp. from different rivers of the Amazon Basin, specimens of *S. discus* collected from the Unini River were examined. This study supplements the original description of the cnidarian myxosporean *C. amazonensis*, providing new data on the stages of and morphological variation in myxospores, thereby extending the original description of the species. Furthermore, differences in myxospore morphology are discussed in the context of intraspecific morphological plasticity.

Materials and methods

In August 2019, thirty specimens of *S. discus* (ranging from 10.3 ± 1.2 cm in total length and 23.4 ± 4.2 g in weight) were collected from the Unini River, near Barcelos Municipality (0°58'30"S, 62°55'26"W), Amazonas State, Brazil. The fishes were sampled under a collection licence issued by the Brazilian Ministry of the Environment (SISBIO Process No. 73241-2). The euthanasia procedure was approved by the Federal University of Amazonas Ethics Committee for Scientific Use of Animals (CEUA-UFAM No. 025/2019). After necropsy, gall bladders were carefully removed and placed in small Petri dishes for further examination under stereo and optical microscopes. Samples of the bile were collected by puncturing the gall bladder using a pointed glass pipette; a drop of bile was then pipetted on to a microscope slide, covered with a cover slip and observed under an Olympus BX53 light microscope at 400× magnification.

Morphological and morphometric analyses were performed on 30 randomly selected mature myxospores using a computer, equipped with Axivision 4.1 image capture software coupled to an Axioplan 2 Zeiss microscope. Following the criteria outlined by Lom and Arthur (1989) and Heiniger et al. (2008), measurements taken for each myxospore included spore length (SL), spore thickness (ST), polar capsule length (PCL) and polar capsule width (PCW) in micrometres (µm) and posterior angle (PA) in degrees (°). The myxospore dimensions were expressed as mean and standard deviation, followed by the range in parentheses. Smears containing free myxospores were air-dried, fixed with methanol and stained with Giemsa to mount on permanent slides. Slides with stained myxospores and vials containing formalin-fixed plasmodia were deposited in the cnidarian collection of the Zoology Museum at the University of São Paulo – USP, São Paulo, Brazil (MZUSP).

For transmission electron microscopy, infected gall bladders were fixed for two days in 2.5% glutaraldehyde, diluted in 0.1 M sodium cacodylate buffer (pH 7.4), washed in a glucose-saline solution for 2 h and post-fixed in 2% osmium tetroxide (OsO_4) for 4 to 5 h. After dehydration in an ascending concentration series of ethanol, the samples were embedded in EMbed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA) (Mathews et al. 2020c). Ultra-thin sections, double stained with uranyl acetate and lead citrate, were examined under a LEO 906 electron microscope operating at 60 kV in the Center for Electronic Microscopy (CEME) at the Federal University of São Paulo.

Genomic DNA (gDNA) was extracted from infected bile of a fish sample and preserved in absolute ethanol. The sample was pelleted through centrifugation at 8,000 rpm for 12 min and the ethanol removed. The gDNA was extracted from the pellet using a DNeasy Blood & Tissue Kit (animal tissue protocol) (Qiagen Inc., California, USA), in accordance with the manufacturer's instructions. The gDNA concentration was quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm. Polymerase chain reaction (PCR) was performed in accordance with Mathews et al. (2016) with a final reaction volume of 25 µl, which comprised 1 µl of DNA (10–50 ng), 0.5 µl of each specific primer (0.2 µM), 12.5 µl of Dream Taq Green PCR Master Mix (Thermo Scientific) and 10.5 µl of nuclease-free water. Partial 18S rDNA sequences were amplified using the universal eukaryotic primer pair ERIB1 (forward: ACCTGGTTGATCCTGCCAG) and ERIB10 (reverse: CTTCCGCAGGTTCACCTACGG) (Barta et al. 1997).

The amplification of the partial 18S rDNA was performed on a Mastercycler nexus (Eppendorf, Hamburg, Germany) and the PCR cycle consisted of an initial

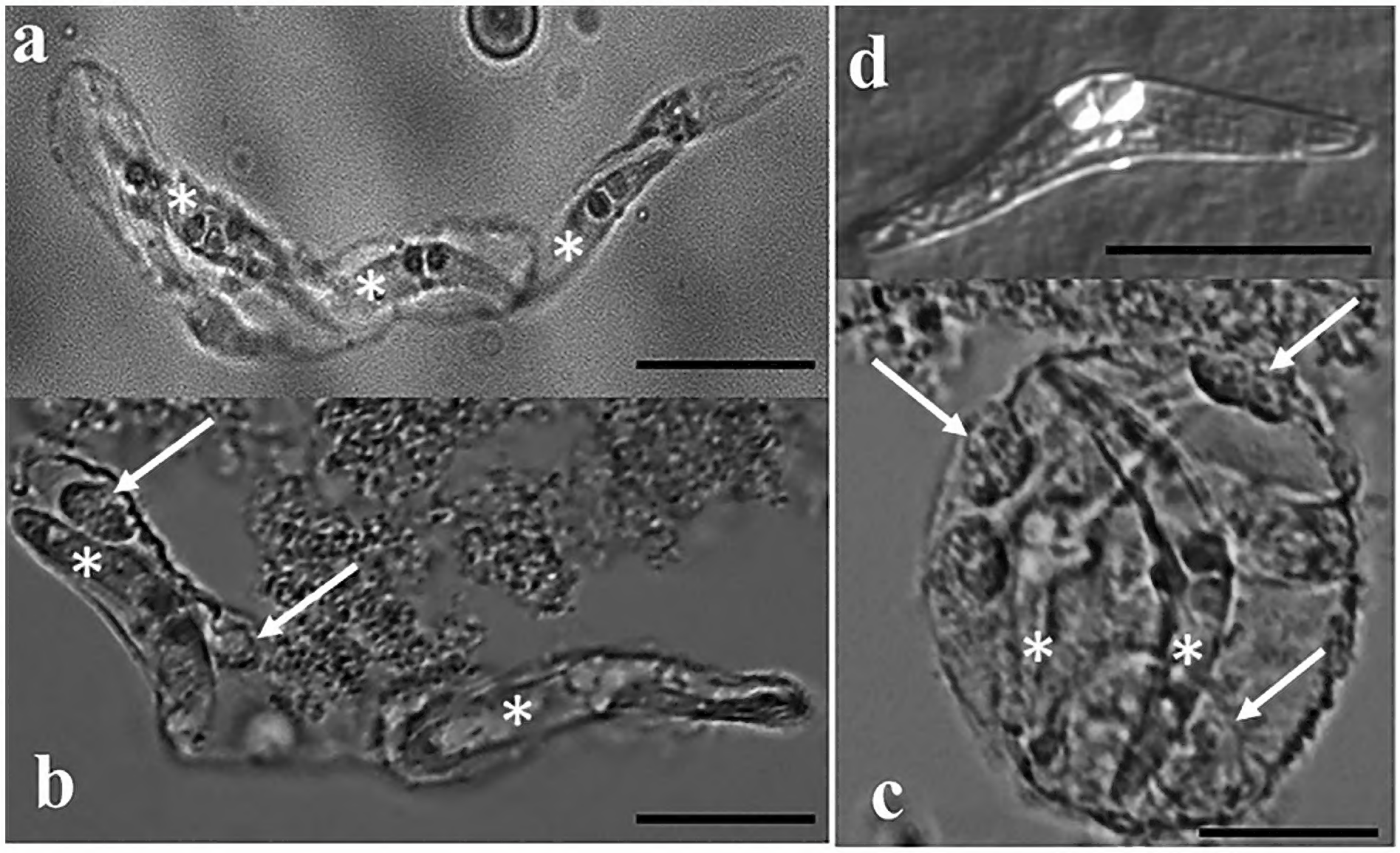

Figure 1. Light photomicrographs of *Ceratomyxa amazonensis* plasmodia. **a, b.** Slightly elongated plasmodia showing mature myxospores (white asterisks) and few early sporogonic stages (arrows); **c.** Spherical plasmodium with two slightly crescent-shaped mature myxospores (ms) and containing early sporogonic stages (arrows); **d.** Differential interference contrast microscopy snapshot of a slightly crescent-shaped mature myxospore. Scale bars: 10 µm.

denaturation step at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, with a terminal extension at 72 °C for 5 min. A control reaction was processed in order to check for possible contamination. The amplified PCR product was subjected to electrophoresis on 1.0% agarose gel (BioAmerica, California, USA) in a TAE buffer (Tris–Acetate EDTA: Tris 40 mM, acetic acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Invitrogen by Life Technologies, California, USA) and then analysed with a Stratagene 2020E trans-illuminator. For sizing and approximate quantification of PCR product, 1 Kb Plus DNA Ladder (Invitrogen by Life Technologies, USA) was used. The PCR product was purified on a USB ExoSap-IT (Thermo Fisher Scientific, Massachusetts, USA) in accordance with the manufacturer's instructions and sequenced using the PCR primer pair, as well as the additional primer pair MC5, CCTGAGAAACGGCTACCACATCCA and MC3, GATTAGCCTGACAGATC ACTCCACGA (Molnár 2002). This additional primer pair was used in the sequencing to connect the overlapping fragments. Sequencing was performed with a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems Inc., California, USA) on an ABI 3730 DNA sequencing analyser. The sequences obtained were visualised, assembled and edited in BioEdit 7.1.3.0 (Hall 1999) to produce a consensus sequence. A basic local alignment search (BLASTn) was performed to evaluate the similarity of our sequence with other myxosporean sequences available in the NCBI database (Altschul et al. 1997). The newly-acquired 18S rDNA gene sequence was aligned with all available Amazonian *Ceratomyxa* spp. sequences, in order to evaluate pairwise genetic distance using the p-distance model in MEGA 6.0 (Tamura et al. 2013).

Results

Freely-floating plasmodia were found in the gall bladder bile of 18 (60%) out of the 30 *S. discus* specimens collected in the Unini River. After rupturing the plasmodia, slightly crescent-shaped mature myxospores were observed with sub-spherical polar capsules. These capsules were located close to the myxospore suture line in a plane perpendicular to it, at the anterior myxospore pole, thus defining classification within the genus *Ceratomyxa*. No signs of infection were observed in the parasitised organs.

Description

Plasmodia were asymmetric and slightly elongated, with mean length 62.3 (range 58.4–64.2) µm and mean width 7.8 (range 6.6–8.9) µm; they contained both mature myxospores and early sporogonic stages (Fig. 1A, B). Some

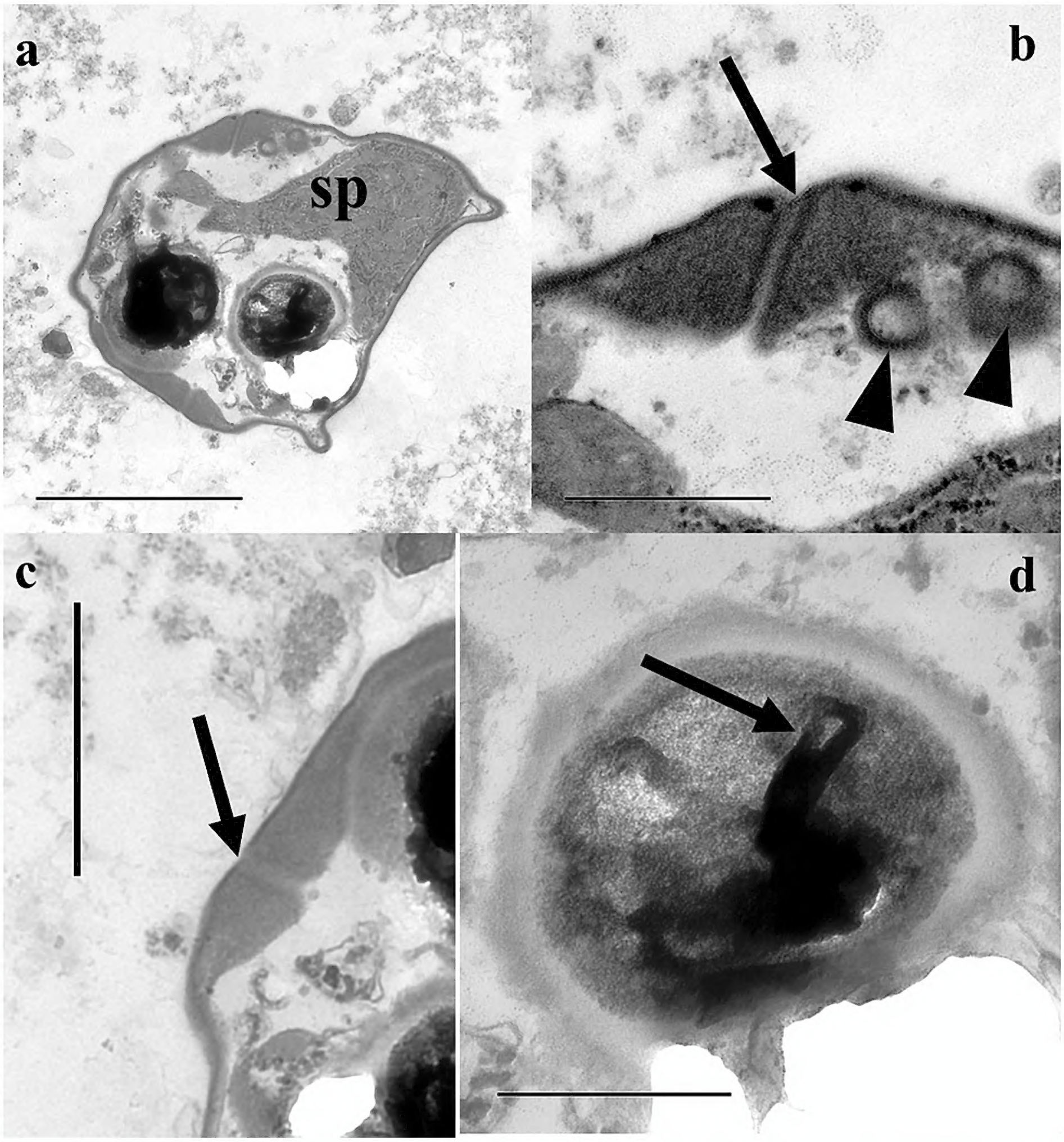

Figure 2. Transmission electron microscopy images of *Ceratomyxa amazonensis* isolated of *Symphysodon discus* from the Unini River, Amazonas State, Brazil. **a**. Myxospore showing two sub-spherical polar capsules and sporoplasm (sp) occupying most of the myxospore volume; **b.** Detail of the apical suture (black arrow) and sporoplasmosomes (arrowheads); **c.** Detail of lateral suture (black arrow); **d.** Polar capsule displaying still uncoiled internal polar tubule (black arrow). Scale bars: 2 µm (**a**); 1 µm (**c**); 500 nm (**b**, **d**).

plasmodia were spherical, mean diameter 30 (range 27–32) µm, n = 11, containing both mature myxospores and visible sporogonic stages (Fig. 1C). Mature myxospores were slightly crescent-shaped, measuring 4.72 ± 0.1 (4.52–4.81) µm in length and 24.2 ± 0.4 (23.9–25.3) µm in thickness (Fig. 1D). Shell valves were approximately equal in size with rounded extremities (Fig. 1D). Apical and lateral sutures were noticeable at the junction of the valves with fine material between them (Fig. 2B, C). The posterior angle was 154° (153°–156°). Polar capsules were equal in size, sub-spherical, measuring 2.31 ± 0.1 (2.29–2.33) µm in length and 2.15 ± 0.1 (2.13–2.17) µm in width (Figs 1D and 2A) and displayed a still uncoiled internal polar tubule (2D). Sporoplasm occupied most of the myxospore volume (Figs 1D and 2A) with sporoplasmosomes present (Fig. 2B).

Host: *Symphysodon discus* Heckel, 1840 (Perciformes: Cichlidae).

Type locality: Unini River, near Barcelos Municipality (0°58'30"S, 62°55'26"W), Amazonas State, Brazil.

Table 1. Pairwise genetic distance of 18S rDNA sequences from *Ceratomyxa* species described from strictly Amazonian fish hosts. The upper triangular matrix shows the number of different nucleotide positions; the lower triangular matrix shows the percentage of differing nucleotide positions.

Species (GenBank ID)	1	2	3	4	5
1. Unini River isolates (this study) (MN064752)	-	77	48	32	1
2. *Ceratomyxa gracillima* (KY934184.1)	7	-	92	109	109
3. *Ceratomyxa vermiformis* (KX278420.1)	4	5.2	-	82	74
4. *Ceratomyxa brasiliensis* (KU978813.1)	3	6.8	5.1	-	38
5. *Ceratomyxa amazonensis* (KX236169.1)	0.1	6.9	4.7	2.4	-

Sites of infection: Within gall bladder (plasmodia floating free in the bile).

Material deposited: The partial 18S rDNA gene sequence was deposited in GenBank (accession number MN064752). Slides with stained myxospores and vials containing formalin-fixed plasmodia were deposited in the cnidarian collection of the Zoology Museum at the University of São Paulo – USP, São Paulo, Brazil (MZUSP 8469).

Molecular Analysis

The BLAST search revealed a high similarity between the newly-obtained 18S rDNA gene sequence and a previously-published sequence of *C. amazonensis* (query cover 100%, maximum identity 99.91%), a parasite of *S. discus* from Rio Negro River. The pairwise comparison between the new isolate from the Unini River and a previously deposited 18S rDNA gene sequence of *C. amazonensis* found an overall genetic divergence of 0.1% with just a single nucleotide base change between the two sequences (Table 1).

Discussion

As pointed out by Lom and Arthur (1989) in their guidelines for the description of myxosporean cnidarians, it is indispensable to provide as much information as possible about the plasmodial stage, such as the site of infection, structure, shape and size. In contrast to the previous description of *C. amazonensis* from *S. discus* from the Rio Negro River, where only free myxospores were reported (Mathews et al. 2016), in our study, plasmodia containing mature myxospores and early stages were found floating freely in the bile within host gall bladders. Here, we provide new data on the plasmodial stage, extending and thereby improving the original description of *C. amazonensis*.

Morphological plasticity in myxospores are acknowledged to be one of the main factors responsible for the difficulties encountered in myxosporean taxonomy and species identification, resulting in taxonomic dilemmas (Zhai et al. 2016, Guo et al. 2018, Xi et al. 2019). It is widely recognised that *Ceratomyxa* spp. myxospores can display a high degree of morphological plasticity (Atkinson et al. 2015, Bartošová-Sojková et al. 2018); thus, classifications, based strictly on morphology, can result in ambiguous descriptions, especially considering that there is a high level of natural morphological and morphometric variation in myxospores both within and between hosts (Atkinson et al. 2015). One reason for this variation is that myxosporean infections typically feature asynchronous myxospore development, so that changes in shape and size during maturation result in a range of myxospore morphologies (Atkinson et al. 2015). A further problem in using myxospore morphology alone to distinguish species concerns the fixative methods used, because it is known that fixatives can affect the morphological dimensions of myxospores relative to fresh samples (Parker and Warner 1970, Zhai et al. 2016). In our study, no changes were observed in the dimensions of formalin-fixed myxospores compared to fresh samples; this could be explained by the type of fixative used, which is reported to have little, if any, effect on myxospore dimensions (Parker and Warner 1970). These observations are consistent with those reported for *Myxobolus drjagini* Akhmerov, 1954, where myxospores, fixed in 10% formalin since the 1980s, showed little shrinkage compared with fresh myxospores (Xi et al. 2019). However, morphological variations in some *Ceratomyxa* spp. have been described as created by deformations of their presumably thin-walled shell valves (Morrison et al. 1996), thus limiting the use of myxospore features as a sole approach to taxonomic classification. Under this scenario, in order to accurately identify new myxosporeans species, it is highly recommended to use a combination of morphological and biological traits, factors related to host ecology and molecular characteristics, particularly within genera with high intraspecific variation in myxospores, with *Ceratomyxa* being a remarkable example (Atkinson et al. 2015).

In our study, the morphological comparison between the new myxospore isolate from Unini River, *S. discus* and previously described *C. amazonensis* myxospores found in specimens from the Rio Negro River, shows some dissimilar characteristics (Table 2). Unini River myxospores were slightly crescent-shaped, shorter in length and significantly thicker compared to the Rio Negro River myxospores which were strongly arcuate shaped, comparatively longer and less thick (Mathews et al. 2016). Although we noticed some discordance in myxospore morphology between the new isolate obtained and previously described *C. amazonensis* from Rio Negro River, the molecular analysis revealed a high similarity (99.91%) in 18S rDNA sequence data, with only a single

Table 2. Comparative morphometric data for the newly-isolated myxospores and other *Ceratomyxa* parasites of Amazonian fish. T: thickness; L: length; PCL: length of polar capsule; PCW: width of polar capsule; PA°: Polar angle; –: no data. All measurements expressed as mean ± SD.

Species	T	L	PCL	PCW	PA°	Source
New isolate	24.2 ± 0.4	4.7 ± 0.1	2.31 ± 0.1	2.15 ± 0.1	154	This study
C. amazonensis	15.8 ± 0.4	7.0 ± 0.3	3.2 ± 0.3	2.6 ± 0.2	103.7	Mathews et al. (2016)
C. fonsecai	28.9 ± 2.7	2.6 ± 0.1	1.9 ± 0.3	1.7 ± 0.2	164.8	Silva et al. (2020)
C. mylei	24.6 ± 08	5.1 ± 0.2	2.1 ± 0.3	-	-	Azevedo et al. (2011)
C. brasiliensis	41.2 ± 2.9	6.3 ± 0.6	2.6 ± 0.3	2.5 ± 0.4	147	Zatti et al. (2017)
C. gracillima	28.0 ± 3.4	4.4 ± 1.1	1.9 ± 0.4	1.9 ± 0.4	36.6	Zatti et al. (2018)
C. microlepis	35.5 ± 0.9	5.2 ± 0.4	2.2 ± 0.3	2.2 ± 0.3		Azevedo et al. (2013)
C. vermiformis	8.4 ± 0.4	4.5 ± 0.2	2.7 ± 0.1	2.7 ± 0.1	30.2	Adriano and Okamura (2017)

nucleotide base change. Previous studies in several geographic regions have reported different values for 18S rDNA intraspecific divergence in *Ceratomyxa* (Sanil et al. 2017, Bartošová-Sojková et al. 2018) and there is no universal criterion regarding what constitutes a sufficient level of sequence variation to represent distinct species within the genus *Ceratomyxa* (Bartošová-Sojková et al. 2018). However, the low 18S rDNA genetic difference (0.1%) observed between the isolate obtained in the present study and the previously available *C. amazonensis* sequence is not sufficient to designate them as different species, taking into account that 18S rDNA intraspecific sequence variation of < 1% is common (Zhao et al. 2013, Atkinson et al. 2015, Wang et al. 2019). The low genetic divergence observed between isolates from these two widely-separated localities (511 km apart), indicates that geography has had limited impact in terms of genetic differentiation. Our finding is consistent with previous studies where samples of the same myxosporeans species, collected in distant geographic areas, had zero or very low genetic divergence in their 18S rDNA sequences (Urawa et al. 2011, Adriano et al. 2012, Wang et al. 2019). According to Whipps and Kent (2006), host distribution and migration can be equally important factors in maintaining parasite gene flow over broad geographic areas. The high genetic similarity between the Unini River isolate and the Rio Negro River *C. amazonensis* is likely a result of adaptation of the host, *S. discus*, to floodwater habitats of the Amazon Region, ensuring that populations share a continuous habitat across a large geographical area, despite dry periods between the floods. This adaptation to a specific ecological niche may lead to high parasite gene flow over broad geographic areas. For instance, Zatti et al. (2018) observed an absence of genetic variation in the typically more variable ITS-1 region in widely separated *Ceratomyxa gracillima* samples infecting the gall bladder of the Amazonian catfish *Brachyplatystoma rousseauxii* Castelnau, 1855. They suggest high gene flow as a result of panmixia in the parasite populations due to migratory behaviour of the fish host.

Based on the discussion above, we infer that the myxospores, newly isolated from Unini River *S. discus*, should be regarded as belonging to the previously described species *C. amazonensis*. Furthermore, the observations made during this study highlight that classifications, based strictly on morphology, can result in ambiguous descriptions and reinforce the importance of molecular methods (DNA sequencing) for identifying and distinguishing between *Ceratomyxa* species.

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